Heliyon 5 (2019) e02917

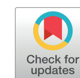
Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Nutrient germination improves DNA recovery from industrial *Bacillus subtilis* endospores during qPCR enumeration assays



John P. Gorsuch *, Peyton Woodruff

BiOWiSH Technologies, 2724 Erie Avenue Suite C, Cincinnati, OH, 45208, USA

ARTICLE INFO

Keywords:
Biotechnology
Microbiology
Molecular biology
Bacillus subtilis
Endospore
Germination
qPCR

ABSTRACT

Growth-independent microbial enumeration methods such as quantitative PCR require the efficient extraction of genomic DNA from targeted cells. *Bacillus* endospores are popular inclusions in commercial products due to their hardiness and metabolic dormancy; however, this hardiness is known to render *Bacillus* endospores resistant to traditional DNA isolation techniques. Metagenomic studies have sought to address this resistance through nutrient-based germination of bacterial endospores in environmental samples. In the present study, we sought to apply this technique to the enumeration of microbial products using an industrial strain of *Bacillus subtilis* as a model organism. Germination was induced through incubation of axenic spore suspensions in an AGFK-based rich medium. Total spore count, dipicolinic acid release and OD_{600} absorbance were monitored over time to track the progression of spore populations through the stages of germination and outgrowth. Aerobic plate counts and flow cytometry were used to monitor cell populations for proliferation during the incubation period. Finally, quantitative PCR with taxon-specific primers was used to examine DNA recovery as a function of time. Results show that customized germination protocols, once appropriately validated for the species and product matrix under consideration, can result in more efficient DNA extraction and thus lower limits of detection for qPCR assays targeting industrial *Bacillus* endospores in microbial products.

1. Introduction

Due to their metabolic dormancy and resistance to environmental and chemical stressors, *Bacillus* endospores are popular inclusions in microbially-based products (Cutting, 2011). Global labeling and regulatory mandates require the accurate enumeration of *Bacillus* endospores in such products, and thus they are routinely subjected to enumeration assays. Growth-based plate counting methods such as the aerobic plate count (APC) are the industry standard for enumerating microbial products; however, even under optimal conditions these assays tend to underestimate microbial concentration (Davis, 2014; Sutton, 2012) and standard iterations are sometimes inappropriate for the enumeration of *Bacillus*-based products (Gorsuch et al., 2019a). Growth-independent enumeration methods such as quantitative polymerase chain reaction (qPCR), digital polymerase chain reaction (dPCR) and flow cytometry (FC) address many of the APC assay's limitations and thus represent attractive alternatives to plate counting (Davis, 2014; Gorsuch et al., 2019b).

In the case of qPCR and dPCR, microbial enumeration cannot be conducted until genomic DNA is extracted and isolated from the cells of interest. This presents a challenge for *Bacillus*-based products, as the same hardiness which makes endospores so attractive to manufacturers also renders many of them resistant to traditional DNA isolation techniques (Lara-Reyna et al., 2000; Filippidou et al., 2015). In an interesting reversal of Staley and Konopka's “great plate count anomaly” (Staley and Konopka, 1985) the resistance of endospores to DNA extraction contributes to the underrepresentation of endospore forming members of the phylum Firmicutes in metagenomic studies (Filippidou et al., 2015). In the presence of nutrient and non-nutrient germinants an endospore will rapidly resume metabolic activity and thus lose its trademark resistance properties (Setlow, 2014). In order to facilitate endospore detection, the authors of some metagenomic studies have exploited the spore's germination response to render them more amenable to DNA extraction and subsequent detection (Lara-Reyna, 2000).

This approach is not without limitations for quantitative microbial detection assays. Upon the completion of germination, *Bacillus* cells undergo the process of outgrowth in which the cell conducts protein synthesis, nucleotide synthesis and, eventually, DNA replication (Paidhungat and Setlow, 2002). In an assay where recovered DNA is used to quantify populations of *Bacillus* cells, the onset of DNA replication

* Corresponding author.
E-mail address: jgorsuch@biowishtech.com (J.P. Gorsuch).

https://doi.org/10.1016/j.heliyon.2019.e02917
Received 8 October 2019; Received in revised form 5 November 2019; Accepted 22 November 2019
2405-8440/© 2019 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

among an appreciable subset of the population could lead to misleadingly high DNA yields from a given quantity of cells. Therefore, it may be advantageous to target DNA extraction when a majority of the *Bacillus* population are early in the process of outgrowth, as the endospores have lost their trademark durability, but have yet to engage in DNA replication.

In order to design a germination protocol which meets these criteria for populations of a given *Bacillus* species, such as the population found in a sample of commercial product, an intimate understanding of how germination unfolds in populations of the targeted organism is essential. Fortunately, though some gaps in our knowledge remain, the process of endospore germination is quite well understood and has been summarized eloquently in textbooks (Paidhungat and Setlow, 2002) and in review articles (Setlow, 2014). Common microbiological methods can be used to assess the progress of an endospore population through the stages of germination. Endospores lose their trademark heat stability early in the germination process, and growth-based Total Spore Count (TSC) assays use pasteurization prior to plate counting to quantify this shift within the population. Endospore cores also contain large reserves of pyridine-2,6-dicarboxylic acid, also known as dipicolinic acid or DPA, which is released upon germination (Setlow, 2014) and can be measured colorimetrically in solution (Janssen, 1958). Decreases in absorbance at OD_{600} for a spore population can be monitored to track the excretion of spore components and core rehydration (Paidhungat and Setlow, 2002) as well as the shift in the refractive index of the endospores during germination (Zhang et al., 2015).

A germination protocol which renders the majority of an endospore population amenable to DNA extraction by inducing germination – and which avoids the onset of appreciable DNA replication within the cell population – may have the potential to improve DNA recovery from *Bacillus*-based products, resulting in lower limits of detection (LOD) for qPCR assays. In the present study, we characterized the onset and progression of germination in an axenic population of industrial *Bacillus subtilis* (BS) and monitored improvements in DNA recovery as germination proceeded using qPCR with taxon-specific primers. We assessed the release of DPA, shifts in OD_{600} absorbance, and the loss of heat stability as a function of time to track the progression of spore populations through the stages of germination. Cell concentration was monitored using APC and FC assays to rule out the onset of cell proliferation. Although nutrient germination protocols are likely to be highly specific to individual *Bacillus* strains and product matrixes, nutrient germination may have the potential to bring the same type of benefit to quality control and regulatory enumeration assays that it has brought to metagenomic studies.

2. Materials and methods

2.1. Preparation of *Bacillus subtilis* endospore suspensions

An industrial strain of *Bacillus subtilis* (BS) used in commercial products (United States patent US 10398156B2) was selected as a model organism for this study. Axenic BS endospore suspensions with concentrations of 1.0×10^{10} CFU/mL were obtained from an industrial fermentation company. Spore suspensions were produced by the supplier using proprietary methods which were not made available to the authors. Prior to use in experiments, BS endospore suspensions were pelleted, rinsed and resuspended in sterile phosphate-buffered saline (PBS, Thomas Scientific, Swedesboro NJ) to remove any background DPA and to dilute germination inhibitors added by the supplier. Centrifugation at 5,000 RCF was carried out at ambient temperature. Supernatant was decanted, replaced with sterile PBS, and the cell pellet was resuspended by aspiration with a sterile serological pipette. Once the pellet was completely resuspended, the process was repeated. Rinsed and resuspended endospore suspensions were stored at 4 °C until needed.

2.2. Preparation of germination medium and batch reactor flasks

Customized germination medium was prepared which included the BS germinant mixture L-asparagine, D-glucose, D-fructose and potassium (AGFK, Paidhungat and Setlow, 2002). Tryptic Soy Broth (TSB, Carolina Biological Supply, Burlington NC) containing D-glucose (2.5 g/L) and dipotassium phosphate (2.5 g/L) was augmented with L-asparagine (Millipore Sigma, Burlington MA) at 3.0 g/L and D-Fructose (Millipore Sigma, Burlington MA) at 2.5 g/L. Medium was dispensed in 194mL aliquots into 500mL Erlenmeyer flasks which were capped with aluminum foil and sterilized in an autoclave at 121 °C, 15 psi for 15 min. Flasks were allowed to equilibrate to room temperature prior to use. Sterile PBS was used as a negative control, and PBS flasks were prepared in the same manner as described for flasks of germination medium.

2.3. Dosing, incubation and sampling of batch reactor flasks

Flasks of germination medium and of PBS ($n = 3$ replicates each per treatment) were dosed aseptically with 6mL of the appropriate rinsed endospore suspension (prepared in 1.1 above) for a final flask volume of 200mL. After the collection of 0-minute samples (described below in 1.4) flasks were transferred to an incubator/shaker and held at 37 °C, 200RPM for the duration of the sampling period. Batch reactor flasks were sampled at 15-minute intervals for 105 min. Incubation time was limited to 105 min to avoid the onset of detectable cell proliferation. Preliminary experiments showed that detectable cell proliferation (defined as an increase of $\leq$20% in culturable cell populations using an APC assay) begins for this species under the tested conditions after 150 min of incubation (data not shown). Six separate samples were collected aseptically at each time point: 1.0mL for APC assay, 1.0mL for TSC assay, 1.0mL for OD_{600} testing, 5.0mL for DPA assay, 1.0mL for FC analysis and 1.0mL for DNA extraction and qPCR. Samples not intended for immediate testing were immediately transferred to an ice bath at 4 °C, where they were held until processing.

2.4. Aerobic plate count assays

Samples of batch reactor flask medium (1.0mL) were collected from each replicate flask ($n = 3$ per treatment) at 15-minute intervals over 105 min for APC assays, which were conducted as described in previous work (Gorsuch et al., 2019a) following a spread-plate technique. Culture medium was Tryptic Soy Agar (TSA) augmented with 0.075g.L bile salts (Millipore Sigma, Burlington, MA) and 0.025 g/L Congo red dye (Carolina Biological Supply, Burlington NC). Serial dilutions were conducted beneath a laminar flow hood using sterile 0.1% peptone blank as the diluent. Due to the reactor flasks' nominal cell concentration of 3.0×10^8 CFU/mL, APC assays had a targeted reading frame of 10^{-5}, 10^{-6} and 10^{-7}. The 100μL plating inoculum constituted the final tenfold dilution of each sample and was spread across the surface of each plate with a freshly autoclaved glass spreader. Plates were allowed to sit face-up to absorb the liquid inoculum for 15 min before being inverted and transferred to a plate incubator, where they were held at 37 °C for 24 h before counting.

2.5. Total spore count assays

TSC assays were conducted in the same manner as described above for APC assays, with the exception that targeted dilution bottles were pasteurized at 80 °C for 15 min in a hot water bath before plating aliquots were collected and spread on agar plates.

2.6. Flow cytometry assays

FC analysis was conducted by Bioform Solutions (San Diego, CA) as described in previous research (Gorsuch et al., 2019b). Samples were

stored in an ice bath prior to shipping and were shipped overnight packed in ice to the Bioform Solutions laboratory where they were immediately refrigerated at 4 °C until testing. For each treatment $n = 3$ reactor flasks, and replicate samples were tested in triplicate such that each time point for each replicate flask represents a geomean of three instrument readings.

2.7. Colorimetric determination of dipicolinic acid

Samples of batch reactor flask medium (5.0mL) were collected from each replicate flask ($n = 3$ per treatment) at 15-minute intervals over 105 min for colorimetric determination of extracellular DPA following the protocol of Janssen (1958). Samples were spun in a centrifuge at 5,000 RCF at ambient temperature. Following centrifugation, supernatant was passed through a 0.2μm syringe filter (Thomas Scientific, Swedesboro NJ) at which point 4.0mL was collected using a serological pipette and dispensed into a 20mL scintillation vial (Thomas Scientific, Swedesboro NJ) for colorimetric analysis. Color change was induced by adding 1.0mL of freshly prepared Janssen's reagent (0.5M acetate buffer containing 1% w/v each of Fe(NH4)2(SO4)2 * 6H2O and L-ascorbic acid, respectively, each sourced from Millipore Sigma, Burlington MA) and measuring OD_{440} absorbance with an Agilent Cary UV-Vis spectrophotometer (Agilent Technologies, Santa Clara CA). The instrument was blanked before OD_{440} readings using a solution of 4.0mL sterile germination medium or PBS (as appropriate) and 1.0mL Janssen's reagent. For each treatment $n = 3$ reactor flasks, and replicate samples were read in triplicate such that each time point for each replicate flask was a geomean of three instrument readings. DPA concentration was calculated using a standard curve developed with serial dilutions of pyridine-2, 6-dicarboxylic acid (Millipore Sigma, Burlington MA). The standard curve produced an equation of $y = 0.0029x + 0.0677$ with a correlation coefficient (R^2) of 0.9998.

2.8. OD_{600} absorbance monitoring

Samples of batch reactor flask medium (1.0mL) were collected from each replicate flask ($n = 3$ per treatment) at 15-minute intervals over 105 min for OD_{600} analysis. Samples were diluted tenfold in 9mL of DI H_2O to ensure that all readings had an absorbance of ≤ 0.2. Absorbance at OD_{600} was assessed using an Agilent Cary UV-Vis spectrophotometer (Agilent Technologies, Santa Clara CA) after the instrument was blanked using a tenfold dilution of the appropriate sterile medium in DI H_2O. For each treatment $n = 3$ reactor flasks, and replicate samples were read in triplicate such that each time point for each replicate flask was a geomean of three instrument readings. Each average value was then multiplied by 10 to account for the initial tenfold dilution of the sample.

2.9. Genomic DNA extraction and qPCR

Samples of batch reactor flask medium (1.0mL) were collected from each replicate flask ($n = 3$ per treatment) at 15-minute intervals for 105 min for genomic DNA extraction and qPCR analysis. Genomic DNA was isolated using a QIAGEN DNEasy Powerlyzer Powersoil kit (QIAGEN, Inc., Germantown MD) following manufacturer's instructions. Samples of purified genomic DNA were immediately subjected to qPCR following the protocol described in previous research (Gorsuch et al., 2019b). Taxon-specific BS probes (Life Technologies Corporation, Carlsbad CA) and primers (Eurofins Genomics LLC, Louisville KY) were designed by the Center for Applications in Biotechnology (California Polytechnic State University, San Luis Obispo CA) as detailed in previous research (Gorsuch et al., 2019b). The forward primer sequence used was CCAACATATAAGACCTCTAC, the reverse primer sequence used was TTATTTCATCCCATCCTGAC and the customized TaqMan probe sequence used was CCCAACCAGCGATCCATAC. qPCR reactions were conducted using a Bio-Rad CFX 96 Deep Well C1000 Touch thermal cycler (Bio-Rad Laboratories, Hercules CA). Reaction conditions were 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 60 °C for 60 s. Recovery of genomic DNA from batch reactor flask samples was assessed through a comparison of quantitation cycle (Cq) values reported by the Bio-Rad CFX 96 Deep Well C1000 Touch thermal cycler.

3. Results

3.1. Cell counting assays (APC, FC and TSC)

APC assays showed a static CFU count over the entire incubation period for both treatments, a trend confirmed by static total cell counts as measured by FC (Figure 1). TSC assays showed a static concentration of heat-stable endospores for the duration of the incubation period for control treatments; however, in reactor flasks of germination medium a steady decline in the concentration of heat-stable endospores presented after 15 min of incubation and continued throughout the incubation period (Figure 1). Data points represent the geomean of three replicates per time point, and error bars represent three standard deviations above and below the geomean.

3.2. Spectrophotometric assays (DPA and OD_{600} absorbance)

OD_{600} absorbance values for BS endospore suspensions in PBS remained stable throughout the incubation period; however, in germination treatments OD_{600} values began to decrease after 15 min of incubation with a maximum loss of OD_{600} absorbance occurring after 60 min of incubation (Figure 2). Colorimetric assays for extracellular DPA show no detectable release of DPA in control treatments during the incubation period; however, in germination treatments an increase in extracellular DPA was detectable after 15 min of incubation with maximum DPA release occurring at 90 min of incubation (Figure 2).

3.3. qPCR assays

Recovery of genomic DNA from batch reactor flask samples was static throughout the incubation period for PBS treatments, while germination treatments showed increased DNA recovery as a function of time before stabilizing at 75, 90- and 105-minute time points (Figure 3).

4. Discussion

Growth-independent enumeration methods such as qPCR are desirable alternatives to the industry-standard APC assay for the enumeration of *Bacillus*-based microbial products; however, the same durability which makes *Bacillus* endospores so appealing to manufacturers can also render them resistant to traditional DNA isolation techniques (Lara-Reyna, 2000; Filippidou et al., 2015). In the present study, we examined DNA recovery from a model industrial strain of *Bacillus subtilis* as a function of incubation time in germinant free PBS and in an AGFK-based germination medium. Germination medium reactor flasks showed decreasing TSC counts, a decrease in OD_{600} absorbance (with maximum loss observed at 45 min, Figure 2b) and an increase in extracellular DPA (with release plateauing around 45 min, Figure 2a), data which compare favorably with expectations of an endospore population undergoing germination. PBS reactor flasks showed stable TSC counts and OD600 absorbance as well as undetectable levels of extracellular DPA for the duration of the incubation period (Figure 2), data which do not support the onset of germination. qPCR analysis showed that DNA recovery was improved as a function of time and compared more favorably across replicate flasks of germination medium relative to flasks of PBS (Figure 3), with maximum recovery achieved between 75-105 min. No increase in cell count was detected in either treatment by APC assay or by FC (Figure 1) ruling out the possibility that the improvements in DNA recovery observed between 75-105 min in flasks of germination medium were attributable to cell proliferation.

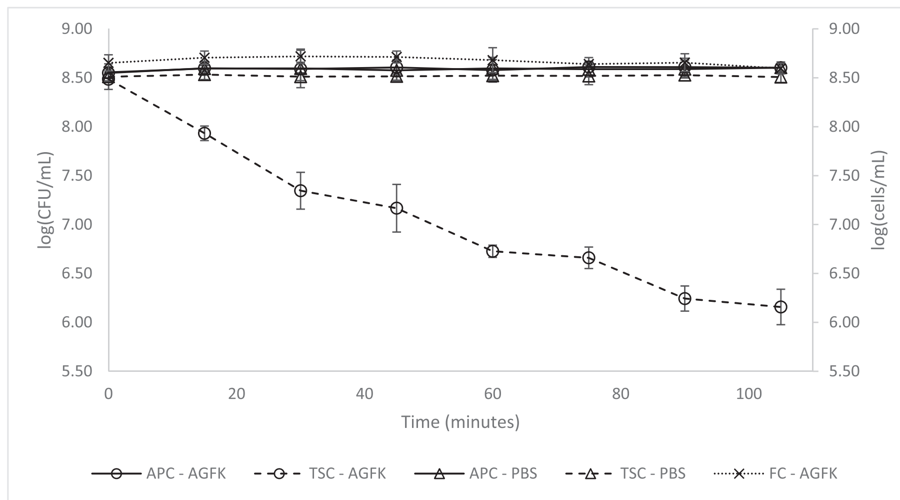


Figure 1. Cell counting assays show a lack of cell proliferation in PBS and AGFK treatments by growth-dependent APC assay as well as growth-independent FC. TSC assays show BS endospores in PBS retaining their heat stability for the entire germination period; however, BS endospores in AGFK continually lose heat stability as a function of time. Error bars represent 3 standard deviations of a geomean above and below (n = 3 replicates per data point).

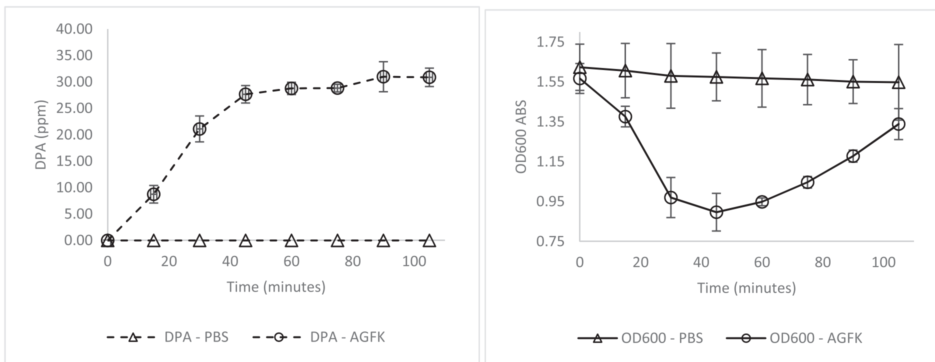


Figure 2. Spectrophotometric analysis of extracellular DPA concentration (a) and OD_{600} absorbance (b) show that extracellular DPA and OD_{600} absorbance are stable over time in PBS reactor flasks; however, extracellular DPA increases as a function of time before stabilizing around 45 min of incubation in flasks of AGFK-based germination medium and OD_{600} absorbance decreases before gradually rebounding. Error bars represent 3 standard deviations of a geomean above and below (n = 3 replicates per data point).

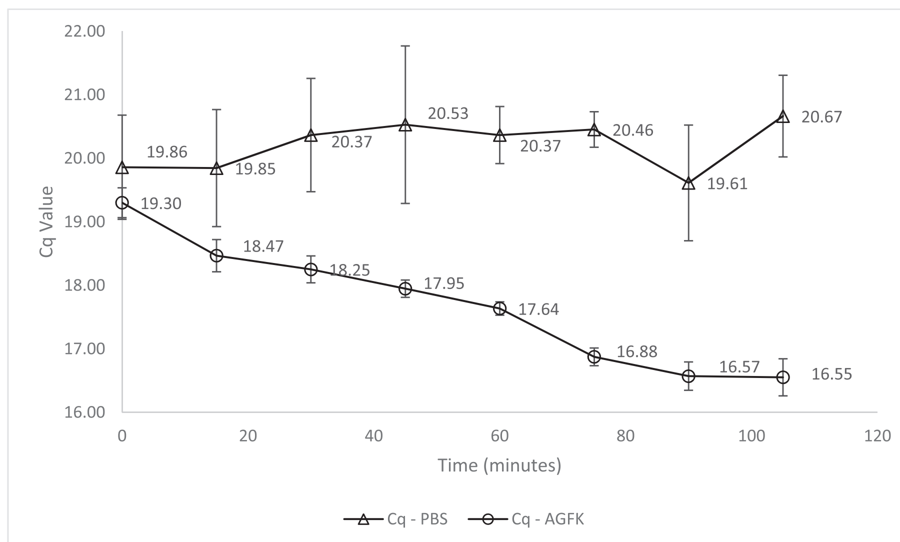


Figure 3. Cq values resulting from qPCR assays with BS-specific primers. DNA recovery was lower (indicated by higher Cq values) and more variable in PBS reactor flasks than in flasks of AGFK-based germination medium. In germination flasks, Cq values plateaued between 90 min and 105 min. Error bars represent standard deviation of a geomean above and below (n = 3 replicates per data point).

It must be noted that none of the methods used here can empirically rule out the replication of genomic DNA as a lurking variable contributing to improved DNA recovery; however, our data would suggest that this interpretation is unlikely to be the case. Data from germination reactor flasks show a maximum loss of OD600 absorbance and a maximum release of DPA occurring for BS endospore populations after 45 min of incubation (Figure 2), suggesting that a majority of BS endospores had not become metabolically active until this point. Continuous increases in OD_{600} absorbance after the occurrence of this minimum paired with a lack of cell proliferation (Figure 1) would suggest the ongoing transition of freshly germinated BS endospores into larger vegetative cells. Furthermore, nucleotide biosynthesis does not generally commence until 10–20 min into outgrowth (Paidhungat and Setlow, 2002). Assuming that a majority of the BS population entered outgrowth at 45 min of incubation, nucleotide biosynthesis would not be expected to commence until 55–65 min. As the largest decreases in Cq value for BS populations in germination medium occur between 0 - 15 min and between 60-75 min, respectively, the appreciable onset of genomic DNA replication seems an unlikely explanation for the trends observed here.

The impact of germination upon DNA recovery from BS endospores suggests that standard curves developed using a germination protocol could have limits of detection (LOD) nearly one order of magnitude lower than standard curves generated without a germination step. Such improvements may allow for the application of qPCR-based enumeration methods to low-activity microbial products. Concentrated endospore preparations such as the BS material described here are often blended into animal feed additives, and then further diluted when such additives are blended into animal feeds, presenting the risk of final *Bacillus* cell counts below the lower LOD of a qPCR assay. Improvements in DNA extraction efficiency may help to alleviate such concerns, allowing the application of PCR-based enumeration methods to a wider variety of microbial products.

For a variety of reasons, we do not propose that the germination medium and timeframe described here represent a broadly applicable approach for improving DNA extraction from industrial *Bacillus* endospores. Resistance to DNA extraction and germination profiles of industrial *Bacillus* endospores are likely to vary considerably from strain to strain, and finished product matrices are likely to be equally diverse. This approach would also require extensive validation for mixed-species *Bacillus* assemblages, as compounds such as DPA released by a germinating endospore can act as non-nutrient germinants for neighboring spores (Paidhungat et al., 2001) raising the possibility that a strain's germination profile may differ when the organism is blended into a mixed-species assemblage. Therefore, it is likely that any germination-based protocol for improving DNA recovery will require extensive validation and optimization for the strain and matrix under consideration. However, our data show that an overall strategy of "germinate to enumerate" could join the well-known "germinate to exterminate" as a means for mitigating the trademark resistance properties of the bacterial endospore, and that such methods may be worthy of consideration during the development of next-generation enumeration methods for *Bacillus*-based products.

Declarations

Author contribution statement

John P. Gorsuch: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Peyton Woodruff: Performed the experiments; Analyzed and interpreted the data.

Funding statement

This work was supported by BiOWiSH Technologies.

Competing interest statement

Both authors are employees of BiOWiSH Technologies, which funded the experiment.

Additional information

No additional information is available for this paper.

References

Cutting, S., 2011. Bacillus probiotics. Food Microbiol. 28, 214–220.
Davis, C., 2014. Enumeration of probiotic strains: review of culture-dependent and alternative techniques to quantify viable bacteria. J. Microbiol. Methods 103, 9–17.
Filipidou, S., Junier, T., Wunderlin, T., Lo, C., Li, P., Chain, P., Junier, P., 2015. Under-detection of endospore-forming Firmicutes in metagenomic data. Comput. Struct. Biotechnol. J. 13, 299–306.
Gorsuch, J., Jones, Z., Le Saint, D., Kitts, C., 2019a. Enumeration of industrial Bacillus assemblages in commercial products with customized plate-counting assays. J. Microbiol. Methods 164, 105682.
Gorsuch, J., Le Saint, D., VanderKelen, J., Buckman, D., Kitts, C., 2019b. A comparison of methods for enumerating bacteria in direct fed microbials for animal feed. J. Microbiol. Methods 160, 124–129.
Janssen, F., Lund, A., Anderson, L., 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science 127, 26–27.
Lara-Reyna, J., Olalde-Portugal, V., Olmedo-Alvarez, G., 2000. An efficient procedure for the isolation of PCR-competent DNA from Bacillus endospores germinated in soil. World J. Microbiol. Biotechnol. (16), 345–351.
Paidhungat, M., Ragkousi, K., Setlow, P., 2001. Genetic requirements for induction of germination of spores of Bacillus subtilis by Ca2+-dipicolinate. J. Bacteriol. 183, 4886–4893.
Paidhungat, M., Setlow, P., 2002. In: Sonenshein, A.L., et al. (Eds.), Spore Germination and Outgrowth. Bacillus Subtilis and its Closest Relatives: from Genes to Cells. ©2002 ASM Press, Washington, DC.
Setlow, P., 2014. Germination of spores of Bacillus species: what we know and do not know. J. Bacteriol. 196, 1297–1305.
Staley, J.T., Konopka, A., 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. 39, 321–346.
Sutton, S., 2012. The limitations of CFU: compliance to CGMP requires good science. J. GXP Compliance 16, 74–80.
Zhang, J.L., Cheng, W.F., Liu, B., Deng, X., Han, N.X., Xing, F., 22–24 June 2015. Rapid quantification of viable spore by a simple spectrophotometric method. In: Proceedings of the Fifth International Conference on Self-Healing Materials. Durham, NC, USA.